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Antioxidant and Anti-Proliferative Activity of *Calamintha officinalis* Extract on Breast Cancer Cell Line MCF-7

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ABSTRACT

The aim of this study was evaluation of antioxidant and anti-proliferative activities of the *Calamintha officinalis* Moench (COM). *Calamintha officinalis* is a genus of aromatic herbs, which is native in north parts of Iran. Some of its pharmaceutical profits are already known, however, a scientific evidence seems necessary before any proposal for its pharmaceutical arrangements. Total antioxidant activity of the plant extract was assayed by spectrophotometric methods. Folin-ciocalteu reagent assay was used to evaluate the phenolic contents of extract. A zymogram assay of peroxidase (POX) was performed to observe its activity. Antiproliferative activity of the plant extract was examined on human breast cancer cell line (MCF-7) using [3-(4, 5-dimethylthiazoly)-2, 5-diphenyl-tetrazolium bromide (MTT) method. The results showed a considerable antioxidant activity and a high reducing power. On the other hand, a reasonable antiproliferative role played by the extract, the POX in this plant is composed of two isozymes. The results indicated that COM can be regarded as a suitable candidate for designing anticancer pharmaceutical preparations.

Key words: Antioxidant, anti-proliferative, *Calamintha officinalis*, breast cancer, MCF-7

INTRODUCTION

Breast cancer, is a common form of cancer being treated usually by chemotherapeutic agents. In most parts of the world, it is placed among the first high percent of female cancers. Breast cancer is also the aim of many research works on search for understanding cancer mechanism, early diagnosis, drug discovery and design of remediation. It is supposed that, if diagnosed at early stages, it can be totally treated. Now-a-days, many herbal preparations have attracted many researchers' attention for cancer treatments. A number of medicinal plants and their bioactive compounds have been shown anticarcinogenic and antiproliferative effects on breast cancer cells.

The use of plant extracts has a long term history in traditional at many countries. Many traditional herbs have

been known for remediation of simple disorders to serious and dangerous diseases including different kinds of cancer ans (Doudach *et al.*, 2013; Fattahi *et al.*, 2013; Ling *et al.*, 2013). It is well known that medicinal plants are potential sources of natural antioxidants (Dragovic-Uzelac *et al.*, 2010; Rafat *et al.*, 2010; Dhal *et al.*, 2012).

The main benefits of using natural formula are their minor side effects, inexpensive and easy accessibility as compared to chemical drugs. The use of medicinal plants for cancer treatment is improving fast in the last few years. Plant extracts contains materials such as flavonoids and aromatic compounds, can be effective in decreasing the oxidative stress presents in cancerous cells (Wang *et al.*, 2015).

Calamintha officinalis is a member of Lamiaceae family and very similar to the common mint from morphological point of view and aroma characteristics. It is generally used as

a substitute for the official mints in various beverages (Monforte *et al.*, 2011; Verma *et al.*, 2011; Singh *et al.*, 2012). Although, COM has been traditionally used since ancient times (Monforte *et al.*, 2012), a scientific research is rarely reported on its characteristics. In the present study, we investigated the antioxidant and antiproliferative activities of the aqueous extract of COM on MCF-7 cell lines in breast cancer. On the other hand, Total Phenolic Content (TPC), Ferric Reducing Antioxidant Power (FRAP) and the activity of POX in its aqueous extract were evaluated.

MATERIALS AND METHODS

Plant samples: The plant, COM was collected from north area of Iran (Gilan, Lahijan). The fresh leaves were washed with distilled water thoroughly and dried at 40°C. The leaves were then crushed into small pieces, frozen and used for extraction.

Preparation of plant extract: Frozen leaves (1 g of fresh mass) were ground in liquid nitrogen and extracted with a cool extraction buffer 3 mL (50 mM potassium phosphate, pH 7.5). The extract was centrifuged for 30 min at 12,000 rpm at 4°C and the resulting supernatants was used as crude extract.

FRAP assay: In this part of experiment, five grams of powdered leaves were added to 50 mL ethanol and mixed thoroughly. It was then placed on ultrasonic bath at room temperature and sonicated constantly for two periods of 5 and 10 min. The resulting mixture was shaken using an incubator shaker at 80 rpm, 25°C for 24 h. The sample was then centrifuged at 3000 rpm for 15 min. The supernatant was used for FRAP assay based on a procedure described by Benzie and Strain (1996). Creation of tripyridyl-S-triazine (TPTZ) complexes with Fe²⁺ [TPTZ-Fe (II)] by a reductive agent existing in reaction mixture led to an increase of absorbance (OD) which was measured at 593 nm. The FRAP reagent contained 2.5 mL of TPTZ solution (10 mmol L⁻¹) in hydrochloric acid (40 mmol L⁻¹) and 2.5 mL of FeCl₃ solution (20 mmol L⁻¹) mixed with 25 mL of acetate buffer (0.3 mol L⁻¹, pH = 3.6). In a reaction mixture, 2.08 mL of FRAP reagent was mixed with 240 µL of water and 80 µL of the properly diluted samples and with standard solution of (FeSO₄·7H₂O 0.3 mM). The mixtures were remained 5 min at room temperature. The absorbances of standard and sample were measured at 593 nm. The FRAP values, were calculated using the calibration curve achieved from standard solution of FeSO₄·7H₂O at 593 nm.

Determination of TPC: The method of Eghdami and Sadeghi (2010) was used with minor modification. In this experiment, 2.5 g of the powder was mixed with 25 mL of 96% methanol. The solution was put on incubator shaker at 80 rpm, 25°C for 48 h. The sample was then filtered on Whatman No. 3 filter paper. Twenty microliter of the plant extract was added to 100 µL of 2 N Folin-Ciocalteu (F-C) reagent. The final volume

of mixture was made to 1600 µL using distilled water. In the last stage, 300 µL of sodium carbonate solution (0.2 mg mL⁻¹) was added to reaction mixture and incubated at 37°C for 45 min. The OD of the solution was later measured at 760 nm. Total phenolic contents was determined as a Gallic Acid (GA) equivalent using a standard curve of GA (ranging from 0.5-2.5 mg mL⁻¹) and expressed as milligram GA per gram of dry sample.

Zymogram analysis: Electrophoresis of enzymatic extract of plant was done by a 12% polyacrylamide gel using the method described by Sharma *et al.* (2013) with some minor modification. Then resulting gel was washed three times in 50 mM (pH 7) phosphate buffer, immersed in a mixture of 0.1 mM phosphate buffer (pH 6.8), guaiacol (30 mM), H₂O₂ (30 mM) and left at room temperature in dark until the formation of reddish-brown bands (Sharma *et al.*, 2013).

POX assay: Activity of POX was measured using a modification of the method described by Chance and Maehly (1955). In practice, the enzymatic reaction was performed by mixing (1.5 mL) 0.1 M phosphate buffer (pH 6.80), guaiacol (30 mM), H₂O₂ (30 mM) and 0.15 mL of enzyme extract. Alternations in Optical Density (OD) of reaction mixture at 470 nm were determined every 20 sec. One unit of POX activity was defined as an OD change of 0.01 unit min⁻¹. The activity of each enzyme was expressed on a protein basis (Rached-Kanouni and Alatou, 2013).

Cell culture: Human breast cancer cell lines (MCF-7 cells) were purchased from Iranian Biological Resource Center (IBRCTM, Tehran, Iran). They were first grown in Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F12 (Sigma, USA) containing 10% heat inactivated Fetal Bovine Serum (FBS) (Invitrogen, South America) and 1% penicillin/streptomycin (Invitrogen, South America). They were then incubated in a 25 cm² culture flasks at 37°C in a humidified incubator set at 5% CO₂. By attaining approximately 70-80% confluency, they were sub-cultured by trypsinization and centrifuged (1000 g, 10 min). The cells were then re-suspended in the culture medium and in exponential growth phase were used for the MTT assay (Doudach *et al.*, 2013).

Determination of cell viability by MTT assay: The MTT colorimetric assay described by Baharum *et al.* (2014) was used to investigate anti-proliferative activity of the extracts against MCF-7 breast cancer cell. In practice, MCF-7 cells were seeded in 96 well plates (density of 10×10³ cells/well for 24 h. The medium was later replaced with fresh one containing different concentrations of extract (1, 5, 10, 25, 50 and 100%) and incubated for another 24 h. In the next stage, 10 µL MTT (5 mg mL⁻¹ in PBS) was added to each well and the plate was incubated for 4 h. To totally dissolve the resulting formazan crystals, 150 µL of DMSO was finally added to each well and the absorbance was read at 570 nm using ELISA microplate reader (BioTek, USA). The IC₅₀ value,

i.e., the concentration of chemical needed to reduce 50% of absorbance relative to control, was then calculated.

Statistical analysis: Statistical analyses were performed using SPSS statistical software and $p < 0.05$ were considered as statistically significant. Results were analyzed using analysis of variance (ANOVA) followed by Dunnett test and expressed as Mean \pm SD. Each concentration was assayed in triplicates ($n = 3$).

RESULTS

Total Phenolic Content (TPC): The TPC of the plant extract was calculated using the calibration curve of GA and presented as GA equivalents per gram (Fig. 1). The resulting TPC value for COM was $0.75 \pm 0.01 \text{ mg g}^{-1}$ indicating that the examined plant extract had significantly high content of phenolic compounds.

Ferric Reducing Antioxidant Power (FRAP): In FRAP test, the OD was measured at 593 nm, the maximum OD for the complex formed from ferrous and TPTZ reaction. The presence of any compound which have any OD in this wavelength can affect the results of FRAP test. The results showed that ethanolic extracts of COM possesses significant ferric reducing antioxidant power. The FRAP antioxidant capacity value for COM was 1.7 mM. This was obtained using a plot of OD at 593 nm from a series of standard solutions with known concentrations (Fig. 2).

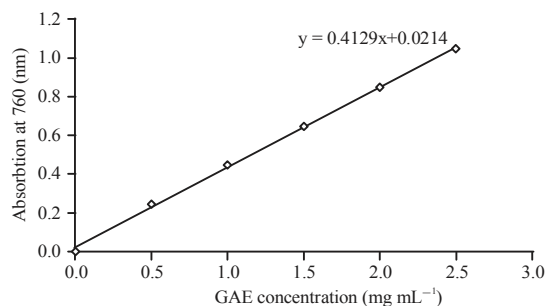


Fig. 1: Standard curve for gallic acid (TPPC)

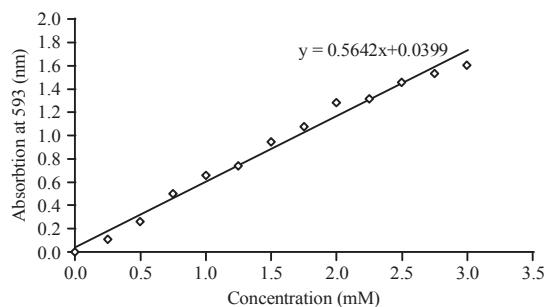


Fig. 2: Standard curve for $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (FRAP) with $\frac{1}{4}$ dilution

Peroxide (POX) activity: Biological activity of POX in extract of COM leaves showed gradual increase with a relative sharp gradient during time (Fig. 3).

The results of all antioxidant tests in present study indicated that COM is a potent source of natural antioxidants (Table 1).

Zymogram: Based on our literature search, COM has not been investigated previously for POX through zymography. In the zymogram test, POX activity was observed as a brown color band and two separate bands were also observed, indicating at least two isoenzymes for POX (Fig. 4).

MTT assay: The MTT assay was used to assess the growth inhibitory effect of leaf extract of COM on MCF-7 cells. The results showed that treatment with different concentrations of the extract significantly inhibited the growth of cancer cells in a concentration-dependent manner (Fig. 5). The inhibitory rates of MCF-7 proliferation at 24 h were 68% for 50 and 55% (V/V) and for 10% (V/V) of extract. In addition, the IC_{50} value of extract was found to be 6% (V/V).

Table 1: Antioxidant activity of COM obtained from different assays

Extract	TPC (mg g^{-1})	FRAP (mM)
<i>Calamintha officinalis</i> extract	0.75	1.7

TPC: Total phenolic contents, FRAP: Ferric reducing antioxidant power

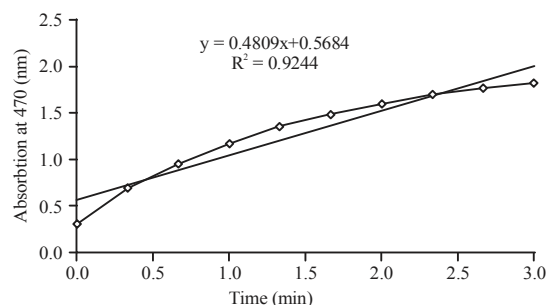


Fig. 3: Activity of peroxidase as a function of time in $\frac{1}{10}$ dilution



Fig. 4: Zymogram of peroxidase from *Calamintha officinalis* moench extract

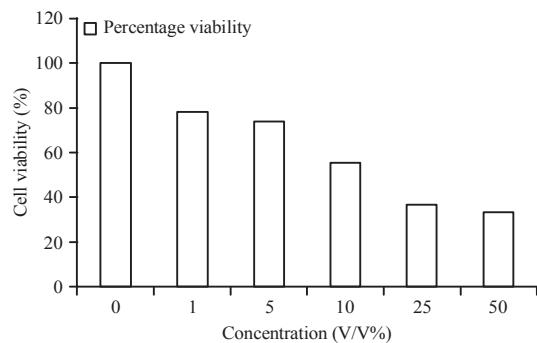


Fig. 5: Dose-dependent antiproliferative effect of aqueous extract of *Calamintha officinalis* moench on MCF-7 cell line

DISCUSSION

Despite many new discoveries on cancer mechanism and drug design, it is expected that the incidences will increase in future (Bray *et al.*, 2012). The search for a natural and inexpensive therapy to prevent, diagnose and inhibit cancer development is becoming an important and novel area. In the recent few decades, human cancer cell lines have been used for evaluation of antiproliferative effect of herbal extracts. The use of cell lines obtained from tumor has the advantage of investigating tumor cells in a simple and controlled environment (Green, 2003). The effect of natural products on cancer is highly dependent on their activity against free radicals, i.e., their antioxidant effect.

In this study, therefore, antioxidant activity of COM extract was first evaluated by FRAP and TPC assays. Based on the results, methods confirmed high antioxidant activity of the extract.

It has been reported that extract of plants such as *Vertiveria*, *Broussonetia* and *Phyla* had cytotoxicity effect on MCF-7 cell line (Teoh *et al.*, 2013; Chitra *et al.*, 2014; Kumar *et al.*, 2014). Thus, antiproliferative activity of enzymatic extract of plant in different concentration (1-50%) was studied on breast cancer cell line MCF-7 by MTT assay. The COM extract presented cytotoxicity on MCF-7 cell line. Antiproliferative activity was accompanied with antioxidant activity. It is proved that a probable relevance between antioxidant activity and cancer inhibition of cell growth (Wang *et al.*, 2007). This findings have demonstrated that the extract of the plant have anticancer effect on breast cancer cell and would be a good candidate to be recommended for future development of drug against breast cancer.

Supportive to our results, in 2011 the effect of various doses (0.4 and 0.8 mL kg⁻¹) of COM oil have been studied on gastric ulcers in pylorus ligation in rats, by other researchers in 2011. Their results suggested the uses of COM essential oil on the treatment of gastric ulcer. However, its significant antiproliferative activity against MCF-7 observed in the present study is novel.

The results of our study, however, are supported by other researchers who worked with various plant extracts and different kind of cancer. The study on medicinal plants is highly dependent on the area of research and their traditional plants as well as the prevalence of a special kind of cancer or any other disease. On the other hand, the use of plant extracts on other types of cancer has shown anti-cancer effect of some medicinal plants. In 2013, anti-cancer, anti-inflammatory and anti-microbial activity of some traditional plants of Jordan was investigated (Assaf *et al.*, 2013). Among their tested plants only one extract, *Viscum cruciatum* Sieb, exhibited anti-cancer activity on hematopoietic malignancies, Burkitt's lymphoma and multiple myeloma.

In support of our study on medicinal plants against cancer, a recent research has reported that Fabaceae family members possess preventive and therapeutic potentials against various types of cancers. They demonstrated that the hydroalcoholic extracts of *Ebenus boissieri* barbey has anti-apoptotic and anti-carcinogenic activity against lung cancer cell line A549 (Aydemir *et al.*, 2015).

CONCLUSIONS

Although, many studies have reported significant advantages of herbal medicines in cancer treatments both *in vitro* and *in vivo*, the use of COM extract has received less attention. Based on the results of our study, it can be concluded that the anti-cancer activity of COM extract is partly due to its potent antioxidant effect. It is, therefore, suggested for further investigations in the discovery and design of new therapy for breast cancer and possibly other cancers.

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